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TITLE: Co-expression of the Follicle Stimulating Hormone Receptor and Stem Cell Markers: A Novel Approach to Target Ovarian Cancer Stem Cells

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REPORT DATE: Ù^] c^{ à^¦/ź013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                        Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE		*Form Approved* OMB No. 0704-0188

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1. REPORT DATE September 2013	**2. REPORT TYPE** Annual	**3. DATES COVERED** 1 September 2012–31 August 2013
4. TITLE AND SUBTITLE Coexpression of the Follicle Stimulating Hormone Receptor and Stem Cell Markers: A Novel Approach to Target Ovarian Cancer Stem Cells		**5a. CONTRACT NUMBER** W81XWH-11-1-0623
		5b. GRANT NUMBER W81XWH-11-1-0623
		5c. PROGRAM ELEMENT NUMBEI
6. AUTHOR(S) David W. Schomberg, PhD		**5d. PROJECT NUMBER**
		5e. TASK NUMBER
E-Mail: david.schomberg@dm.duke.edu		**5f. WORK UNIT NUMBER**
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) DUKE UNIVERSITY DURHAM, NC 27705-4677		**8. PERFORMING ORGANIZATION REPORT**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		**10. SPONSOR/MONITOR'S ACRONYM(S)**
		11. SPONSOR/MONITOR'S REPOF NUMBER(S)
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited		

14. ABSTRACT

The purpose of this project is to determine whether the Follicle-stimulating Hormone Receptor (FSHR) is co-expressed with a sufficient number of ovarian cancer stem cell markers to consider it as a new experimental target for novel nanotechnology approaches capable of destroying ovarian cancer stem/progenitor cells (OCSCs). The work scope involves determining whether: 1) ovarian cancer ascites cells co-express the FSHR and several OCSCs (as determined by fluorescence-activated cell sorting [FACS] and RT-PCR), 2) cultures cloned from a single cell demonstrate co-expression and 3) ascites cells/tumor formed in nude mice continue to co-express the FSHR and relevant markers. To date, we have demonstrated that the FSHR is definitively co-expressed with the OCSC markers CD44, CD133, Notch 2, and Nanog (FACS and/or mRNA; replicated), marginally expressed with Notch 3, Oct 4, ALDH1, and LGR5 (require additional replicates) and is not co-expressed with Notch 1 or 4, CD117, or ABCG2.   FSHR /Notch 2-expressing cells in primary culture expressed FSHR mRNA after several generations in an amount consistent with stem cell characteristics. Nude mouse experiments have demonstrated proof-of-principal and are on-going to confirm co-expression *in vitro.* These results are very encouraging and if extended could justify further experimentation to ascertain whether the FSHR might be a realistic target enabling very specific destruction of OCSC

15. SUBJECT TERMS

ovarian cancer stem cells; Follicle-stimulating Hormone receptor; cancer stem cell markers; fluorescence-activated cell sorting (FACS)

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
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INTRODUCTION:

Subject. The project is designed to determine whether or not the Follicle-stimulating Hormone Receptor (FSHR) is co-expressed on the same cell as various candidate ovarian cancer stem/progenitor cell markers (OCSCS).
Purpose. The project's purpose is to determine whether the FSHR is co-expressed with a sufficient number of ovarian cancer stem cell markers to consider it as a new experimental target for novel nanotechnology approaches capable of destroying OCSCs. Our working hypothesis is that a subset of ovarian cancer ascites cells co-expresses the FSHR and several candidate ovarian cancer stem/progenitor cell markers.
Scope. The work scope involves determining whether: 1) ovarian cancer ascites cells co-express the FSHR and a majority of the currently known OCSCs (as determined by fluorescence-activated cell sorting [FACS] and RT-PCR), 2) cultures cloned from a single double-positive cell (+/- exogenous FSH) continue to demonstrate co-expression and 3) ascites cells/tumors formed in nude mice (+/- elevated endogenous FSH) continue to co-express the FSHR and relevant markers. Positive results from these studies would establish that the FSHR is present and maintained along with the expression of several OCSCs. Further, they would provide the physiological basis for further studies to examine whether the FSHR might be a target capable of facilitating very specific destruction of OCSCs, especially in the postmenopausal patient.

BODY:

A. Accomplishments associated with each task outlined in the approved Statement of Work

Description and timeline of the tasks copied from the SOW:

Subtask 1: Experiments testing whether FSHR and the various candidate stem/progenitor cell markers are co-expressed on ovarian cancer ascites cells. This task will involve working with frozen and fresh ascites cells samples, preparing them for FACS and storing the sorted cells for further analyses, i.e., culture for spheroid formation, RT-PCR, immunofluorescence, and injection into nude mice. Timeframe: 1-12 months.

Subtask 2: Develop spheroids in cell culture and tumors in nude mice. Utilizing the FACS cells above, culture to develop spheroids and also inoculate cells into nude mice plus/minus FSH. Then analyze by FACS, immunofluorescence, H&E staining, and the metrics of measuring tumor response. Timeframe: 6-24 months.

B. Itemization of accomplishments to date (addresses both subtasks):

Year 1

1. By FACS analysis, FSHR was expressed in 85.7% of the patients (18 /21).

2. Definitive co-expression of FSHR and OCSCs at the membrane protein level and/or mRNA expression was shown for Notch 2, Notch 3, CD24, CD44, CD133, and Nanog. The level of FSHR expression (per cent positive live, single cells, white blood cells excluded) was consistent with literature ranges of stem/progenitor cell expression (0.1–10.0%).

3. Marginal co-expression -- requiring confirmation with more highly optimized PCR primer sets – was observed for Oct4, ALDH1, and LGR5.

4. Candidates not showing co-expression at the membrane protein and/or mRNA level were Notch 1, Notch 4, CD117, and ABCG2. MyD88 will not analyzed because it is a downstream effector of CD44; nestin remains to be investigated.

5. FSHR mRNA expression by non-cloned cells co-expressing FSHR and Notch 2 (FACS) was maintained over several generations in primary culture. We will still attempt to demonstrate co-expression following monoclonal expansion of co-expressing cells +/- FSH versus non-FSHR-expressing cells +/- FSH.

6. FSHR/Notch 2 co-expressing cells (+/+, "double positives"), FSHR or Notch 2 "single positives" (+/-) and "double negatives" (-/-) were injected in three graded amounts into cycling and castrated female nude mice. At this point (approximately three months after injection), solid tumors surrounded by a significant degree of fluid have formed from two out of the three cell innocula in the Notch-2-single-positive arm of the ovariectomized group. Ascites is not evident in any of the animals at this point.

7. Monoclonal growth and expansion of cells representing the four cell groups mentioned just above has been attempted but without success to date. We will continue these efforts.

8. Spheroid formation will not be used as evidence of stem cell maintenance/ function. Our collaborators have shown that aggregation rather than monoclonal expansion explains ovarian cancer cell spheroid formation *in vitro* to a large extent and that spheroid formation should only be used very cautiously as a functional end point characteristic of ovarian cancer stem cells (Appendix I).

Year 2

1. Our veterinary staff has since determined that the tumors mentioned above (B6) and shown in Figure 3 were in fact lipomas and not solid tissue tumors. Macroscopically, swollen lymph glands but no ovarian-cancer-like solid tumors or ascites were noted in this group.

2. In the next group of animals our *in vivo* protocol did produce solid tumors with classical ovarian cancer-like gross morphology after 5 months in an ovariectomized animal (high circulating FSH) that received Notch 2 single-positive cells (see Figure 4 below and the comparison to human ovarian cancer).

3. After nine months the remaining animals in this group were sacrificed. Gross morphological nodules and lymphoid swellings that looked ovarian-cancer-like were harvested (from liver, kidney, pancreas, mesentery -- noted principally in the ovariectomized group) and analyzed by RT-PCR for FSHR mRNA expression. At the level of sensitivity obtained without cDNA amplification, FSHR mRNA expression was not detected.

4. New technical procedures for FACS analysis were developed and characterized after the supply of the initial ant-FSHR monoclonal antibody (mab 106.105) was exhausted. This is a secondary antibody detection method and it appears to be at least as good as the initial procedure because: 1) it is more stable over time, and 2) more procedures can be performed with a given quantity of this lot of 106.105 mab.

C. Detailed presentation and discussion of above accomplishments including problems encountered:

1. Brief outline of methods. De-identified human ascites cells were thawed from liquid nitrogen storage, pre-incubated with appropriate blocking proteins/sera and incubated with the appropriate fluorescently labeled monoclonal antibodies and isotype controls (all commercially obtained) using dilutions recommended by the manufacturers. Labeled FSHR antibody of sufficient quality was not available commercially so monoclonal antibody provided by Dr. Jim Dias was labeled in our laboratory using an Alexa 647 labeling kit. The labeling procedure presented problems, which were successfully overcome. The primary issue, among others, was the low yield of conjugated antibody (FSHR-Alexa 647) recovered when the column elution procedure was carried out according to the manufacturer's instructions. This eluted material led to several results showing a very low percentage of FSHR$^+$ cells in the IVF positive-control granulosa/luteal cell samples used to validate each labeling procedure. We solved the problem by cutting off the pipet tip, ejecting the gel material plus elution buffer into a microfuge tube and letting the elution buffer and gel interact for a longer time (empirically

determined, four min.). Neutralization buffer was then added and the phases separated by brief high-speed centrifugation. Labeled antibody obtained in this manner has consistently yielded superior results both in IVF and cancer cell samples. The best explanation I can offer is that the affinity of this particular A647-conjugated FSHR monoclonal antibody type (IgG2b kappa) for the gel resin is so high that a longer exposure to the elution buffer is required. FACS analyses were carried out by the Flow Cytometry Core Facilities of the medical center. RT-PCR of FSHR^{+} cells (and others) was accomplished after RNA isolation (InVitrogen's RNeasy RNA isolation kit) followed by first–strand cDNA synthesis. RNA samples were DNase treated. In certain instances with very small numbers of FSHR^{+} cells (~ 3K) we used InVitrogen's PicoPure RNA extraction procedure (including DNase treatment) followed by the Ovation Pico WTA system® to amplify the RNA into a double stranded cDNA product. Real-time PCR using intron-spanning primers was carried out using a BioRad iCycler. Expression levels of FSHR and the candidate markers were normalized to that of GAPDH. Validation criteria included water blanks, no RT, and melt curve analyses. The PCR results provided independent confirmation of the FACS data and also served as a screen for active gene expression of the non-membrane-associated candidate markers that are not amenable to live-cell FACS analysis. These markers will be analyzed by immunostaining methods. Techniques are in the development and validation stage for FSHR to date. Studies *in vivo* with athymic nude mice have been initiated. Twenty-four mice are used for each FSHR/CSC analysis: three each (10-20K, 1-2K, and 100-200 cells) for each "arm" of FACS-separated cell preps (4 arms: FSHR+/CSC+, FSHR+/CSC-, CSC+/FSHR-, and FSHR-/CSC-) and two groups: cycling, and ovariectomized. Cells were sorted by FACS and immediately diluted for IP injection of the various cell groups within an hour of sorting. The mice are then observed over time for solid tumor and/or ascites formation. Recovered ascites cells will be analyzed by FACS and re-injected into a second set of animals to repeat the findings. Additional ascites cells will be processed and frozen in liquid nitrogen and solid tumor tissue stored/frozen in OCT embedding medium for immunohistochemical analysis.

a. Possible problem encounter, *in vivo study, years 1 - 2*. In the first study, ascites did not develop, so ascites cells for FACS analysis were not available. This only represents one animal, but if this is, in fact, the case for the upcoming animals, we will finely dissect the solid tissue, mince, and digest in enzyme-free cell dissociation buffer (Gibco). This material has worked well for us with cultured cell expressing the FSHR and did not destroy the receptor in the process of lifting and dissociating the cells.

b. Problem encounter and resolution, *in vivo study, years 1 - 2*. We exhausted the supply of mouse anti-human FSHR mab 105.106 from our collaborator Dr. J. Dias during the second year of the study. A particular lot was labeled in our laboratory with the fluor Alexa 647 to provide a

labeled primary antibody. As mentioned above in last year's report, the labeling procedure was problematic. It was also an issue when we tried to replace the original with a different lot of 106.105. We therefore decided instead to utilize it as an unlabeled primary with commercially available goat anti-mouse Alexa 647 as the labeled secondary antibody and this procedure is working well for us (see next section).

2. Results and discussion of FSHR and co-expression analyses.
    FACS analysis. As mentioned above, FSHR was expressed by the ascites cells of 18 of 21 patients (85.7%). Even though the patient number is very small, this result confirms and extends the literature findings showing that expression of the FSHR in ovarian cancer patients is 60% or greater (1). By FACS and/or mRNA analyses of 13 candidate CSC markers (Notch 1-4, CD44, CD117, CD133, CD24. Nanog, Oct4, ALDH1, LGR5, and ABCG2), six of the 13 (46.2%) were co-expressed with the FSHR (Notch 2 and 3, CD24, CD44, CD133, and Nanog). These results may be providing evidence that FSHR action ranges across and facilitates several stem/progenitor cell activity pathways. They may also imply that FSHR is co-expressed on multiple types of CSC populations – suggesting it might demarcate an as yet unknown common progenitor. The existence of multiple types of ovarian cancer stem cells has been proposed (2). Appendix 1 Figure 1 illustrates FACS scans of the typical cellular distribution in two patients and that we have noted in virtually all the patients: all the possible combinations are usually but not always present -- double positives, single positives, and double negatives -- although to differing degrees. Taken together with the previous results, this presumably indicates that different markers and marker combinations are present at any given time of cancer cell development and differentiation.

    mRNA analysis. Messenger RNA analysis was used as an independent confirmation of FSHR and CSC membrane protein co-expression demonstrated by FACS analysis. Examples for FSHR, Notch 2, and GAPDH are shown in Figures 2a -2c, respectively.
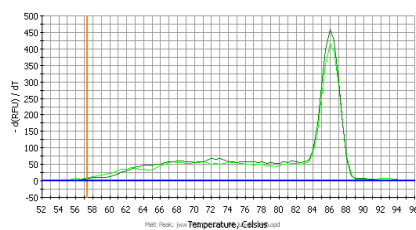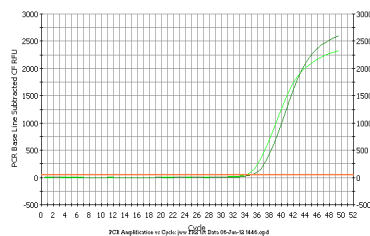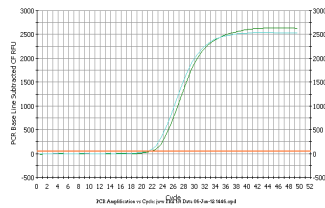   A.   B.

Figure 2a. Real-time analysis of FSHR expression in FSHR/Notch2 double-positive cells determined by FACS analysis. A. PCR quantitation (duplicate samples), B. Melt curves (duplicate samples).

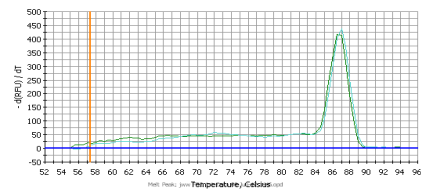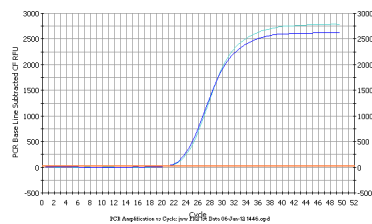A.   B.

Figure 2b. Real-time analysis of Notch 2 expression in FSHR/Notch 2 double-positive cells determined by FACS analysis. A. PCR quantitation (duplicate samples), B. Melt curves (duplicate samples).

A.   B.
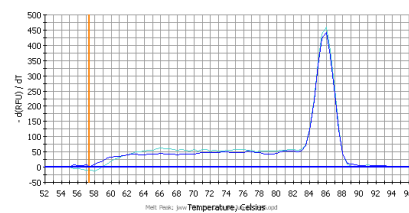

Figure 2c. Real-time analysis of GAPDH expression in FSHR/Notch2 double-positive cells determined by FACS analysis. A. PCR quantitation (duplicate samples), B. Melt curves (duplicate samples).

By these criteria, Nanog mRNA was also demonstrated in FSHR-positive (FACS) cells. Real-time expression and melt curves were also validated for CD24, CD44, CD133, and Notch 3 but specific co-expression each with FSHR mRNA has not yet been accomplished because of limiting cell numbers. Also, Oct4, ALDH1, and LGR5 may qualify as additional markers co-expressed with the FSHR; however, in the first set of analyses the melt curves of their PCR products were not of sufficient quality. Different primer sets may yield acceptable curves and these are being currently tested. Co-expression of some markers were not detected by either FACS or PCR methodology (Notch 1 and 4, CD117) or PCR (ABCG2). For purposes of this investigation these candidates likely will not be examined further.

 FSHR expression by cultured cells. The objective of this study was to determine whether FSHR mRNA expression would be maintained over long-term primary culture. FSHR mRNA expression by non-cloned cells positive for FSHR and Notch 2 (FACS) was maintained over several generations as the cell grew to confluence during two weeks of primary culture. This result is very encouraging because FSHR expression typically is not easily maintained in primary culture of granulosa cells of all species beyond a few days. We will still attempt to demonstrate co-expression following monoclonal expansion of co-expressing cells +/- FSH versus non-FSHR-expressing cells +/- FSH.

Years 1 – 2, tumor growth in vivo: FACS analysis was performed and FSHR/Notch 2 co-expressing cells (+/+, "double positives"), FSHR or Notch 2 "single positives" (+/-) and "double negatives" (-/-) were injected in three graded amounts (100-150, 1,000-1,500, 10,000-15,000 cells, IP) into cycling and castrated female (high FSH) nude mice. At this point (approximately three months after injection), solid tumors surrounded by a significant degree of fluid formed from two of the three cell inocula in the Notch-2-single-positive arm of the ovariectomized group. Ascites is not evident in any of the animals at this point (Figure 3).

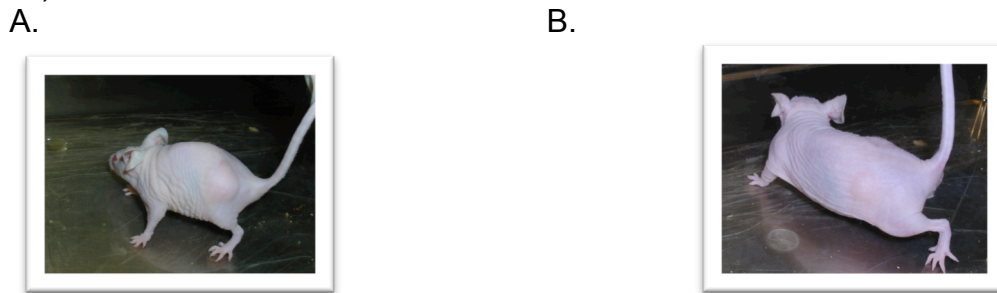A.   B.

Figure 3. Visible subcutaneous tumors developed from injection of Notch 2 single-positive cells: A. 10-,000 – 15,000 cells; B. 100 – 150 cells. They are characterized by a palpable solid mass surrounded by considerable fluid.

According to our hypothesis, the FSHR/Notch 2 double positives should have also formed tumors, and at an even earlier age. However, tumor growth is very variable and we ran this experiment for nine months to allow development of slow-growing tumors.

The tumors shown in Figure 3 were analyzed in more detail in year 2 by our veterinary staff and determined to be lipomas, a condition not uncommon in this particular strain of nude mice. In the repeat of this study no lipomas were noted. However, in this study a mouse in the ovariectomized (presumably high circulating FSH; blood serum was collected for later analysis) group lost weight and died suddenly approximately five months after injection with 1,000 – 2,000 Notch 2 single-positive cells (see Figure 4 below and the comparison to human ovarian cancer).

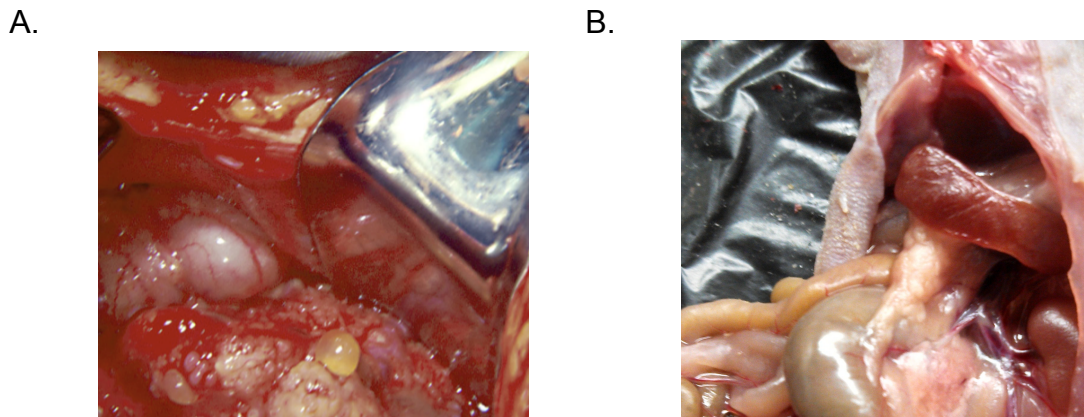A.   B.

Figure 4. Ovarian cancer nodules in human (A) and in a mouse (B) that received Notch 2 single-positive cells.

The morphology of the mouse ovarian cancer-like nodules very closely resembles that of the human. The vascular nature of the mouse tumors appears much less pronounced but that is because the animal had been previously exsanguinated to provide blood serum for possible FSH assay. Tissues have been stored in OCT embedding medium for frozen sections for upcoming immunocytochemical analysis.

 After nine months the remaining animals in this group were sacrificed. Gross morphological nodules and lymphoid swellings that looked ovarian-cancer-like were harvested (from liver, kidney, pancreas, mesentery -- noted principally in the ovariectomized group) and analyzed by RT-PCR for FSHR mRNA expression. At the level of sensitivity obtained without cDNA amplification, FSHR mRNA expression was not detected.

Year 2, development and characterization of a second antibody detection procedure. As mentioned above, the labeling of our primary 106.105 mab was problematic but the issue has been resolved. Table 1 exemplifies that mab amounts and ratios can be empirically determined that accomplish labeling of essentially all the FSHR-positive cells.

Table 1. FACS analysis, characterization of secondary antibody procedure: percent HEK cells expressing FSHR.

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ug/ml goat anti-Mouse A-647	% FSHR$^+$ cells
Isotype control (10.0)	0.25
4.0	73.9
8.0	83.1
16.0	89.0
32.0	88.0
replicate, ug/ml 106.105	
isotype control (10.0)	0.28
2.5	88.1
5.0	92.3
10.0	93.3

Conditions: live, single HEK cells, previously transfected with FSHR including a portion of the extracellular domain; approximately 70,000 FSHR$^+$ cells/tube; HEK cell stock provided by Dr. Jim Dias, U at Albany, SUNY

We now use the combination of conditions that labels approximately 92% of the HEK cells.

Stem cell criteria: monoclonal expansion of co-expressing cells in vitro.
We attempted to demonstrate monoclonal growth and expansion of FACS-separated cells the above cell groups but without success to date. We will continue these efforts. Spheroid formation will not be used as evidence of stem

cell maintenance/function. Our collaborators have shown that aggregation rather than monoclonal expansion explains ovarian cancer cell spheroid formation *in vitro* to a large extent and that spheroid formation should only be used very cautiously as a functional end point characteristic of ovarian cancer stem cells (Appendix 2).

KEY RESEARCH ACCOMPLISHMENTS:

- By FACS analysis, FSHR was expressed in 85.7% of the patients (18 /21).

- Definitive co-expression of FSHR and OCSCs at the membrane protein level and/or mRNA expression was shown for Notch 2, Notch 3, CD44, CD133, CD24 and Nanog. This is six of 13 of the stem/progenitor cell candidate markers analyzed or 46.1%.

- Marginal co-expression -- requiring confirmation with more highly   optimized PCR primer sets – was observed for Oct4, ALDH1, and LGR5.

- Candidates not showing co-expression at the membrane protein and/or mRNA level were Notch 1, Notch 4, CD117, and ABCG2. .

- FSHR mRNA expression by non-cloned cells co-expressing FSHR and Notch 2 (FACS) was maintained over several generations in primary culture.

- Solid tumors surrounded by a significant degree of fluid developed from Notch-2-single-positive cells in ovariectomized nude mice after approximately 2.5 months. (The complete design included FSHR/Notch 2 co-expressing cells (+/+, "double positives"), FSHR or Notch 2 "single positives" (+/-) and "double negatives" (-/-) injected in graded amounts into normally cycling and ovariectomized animals.)

- Ascites is not yet evident in any of the animals at this point.

- Monoclonal growth and expansion of cells representing the four cell groups mentioned just above has been attempted but without success to date.

- Spheroid formation in culture will not be used as evidence of stem cell maintenance/ function.

- An improved detection methodology facilitating FACS analysis has been developed.

- Solid ovarian-cancer-like tumors developed *in vivo* in a nude mice from IP injection of 1,000 – 2,000 Notch 2 single-positive cells. FSHR co-expressing

or FSHR single-positive cells did not develop into tumors in the initial study.

CONCLUSIONS:

Accomplishments. The results to date indicate that several candidate ovarian CSCs – close to one half of the currently suggested candidates – are co-expressed with the FSHR. This is very encouraging and suggests that targeting the FSHR with cytotoxic effectors could simultaneously destroy multiple stem/progenitor cell types and their lineages. The more difficult tasks of demonstrating whether or not the FSHR maintains co-expression with candidate markers following monoclonal expansion *in vitro* and repeat tumor cell inoculation *in vivo* are underway. In terms of proof-of-principle, our initial attempt at monoclonal expansion was unsuccessful but we will attempt several more trials. The *in vivo* study with FSHR and Notch 2 has shown that our *in vivo* protocol is capable of producing ovarian-cancer-like tumors.

Recommended changes going forward. The current approach is satisfactory and no changes in technique or experimental approach are needed. The availability of new patient samples to us has been slow, however, but is beyond our control. Advances in surgical technique and therapy apparently have reduced the number of paracenteses performed by our oncology division and, in turn, the number of sample collections available to us. After a slow period, however, it is possible that a turnaround will occur. As of this writing (9/25/13) it does appear that this is the case. Two patient samples have recently become available.

Significance. To our knowledge, the successful demonstration of co-expression of even a single stem/progenitor cell marker and a specific membrane hormone receptor would be novel and would be the first in the field of ovarian cancer. As mentioned above, successful results in this project could provide justification for the designing of appropriate immunotoxin-based and/or laser-activated conjugates targeting the FSHR.
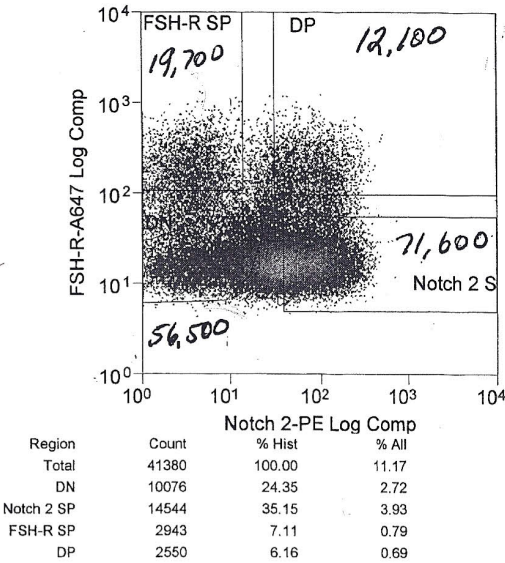
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2. D. Burgos-Ojeda, B. R. Rueda, R. J. Buckanovich, Cancer Letters **322**, 1, (2012)

APPENDICES:

Appendix 1.

All 3 (G3: NOT CD45+ & Cells & Singlets)

Region	Count	% Hist	% All
Total	41380	100.00	11.17
DN	10076	24.35	2.72
Notch 2 SP	14544	35.15	3.93
FSH-R SP	2943	7.11	0.79
DP	2550	6.16	0.69

All 3 (G3: NOT CD45+ & Cells & Singlets)

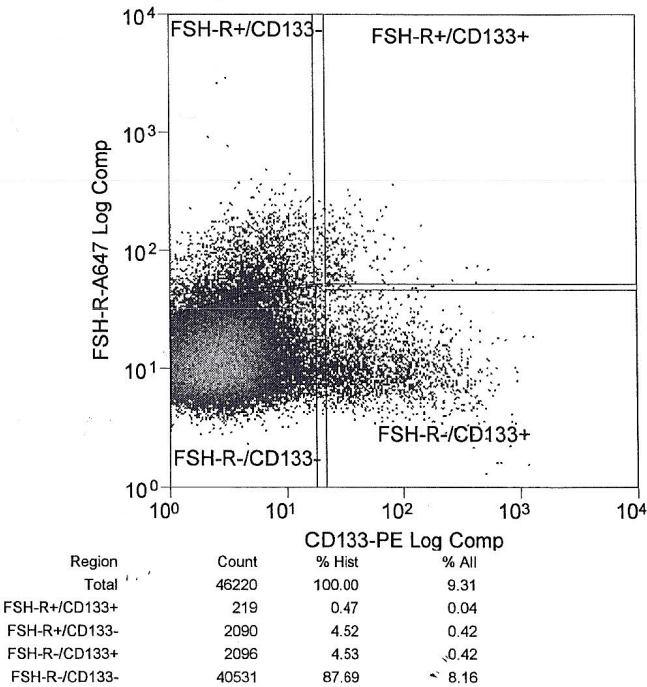| Region	Count	% Hist	% All
Total	46220	100.00	9.31
FSH-R+/CD133+	219	0.47	0.04
FSH-R+/CD133-	2090	4.52	0.42
FSH-R-/CD133+	2096	4.53	0.42
FSH-R-/CD133-	40531	87.69	8.16

Figure 1. FACS analysis (raw data) of ascites cells from two patients showing different degrees of FSHR expression and co-expression with Notch 2 (top) or CD133 (bottom).

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Appendix 2. Copy of the poster text presented at the AACR meeting,
 Chicago, IL, 2012

Aggregation rather than monoclonal expansion explains ovarian cancer spheroid formation

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Purpose: It is thought that a subpopulation of ovarian cancer cells possess stem cell like properties and are responsible for both the emergence of cancer and for subsequent chemoresistance and recurrence. A common approach used to enrich for cancer stems cells *in vitro* involves culture in selective media on low attachment plates resulting in non-attached multicellular spheres. Spheroids are present in ascites fluid of women with ovarian cancer, and may contain stem cells responsible for metastasis and recurrence. Our objective was to address whether spheroids are truly monoclonal in origin and arise from expansion of a single common progenitor cell.

Procedures: We cultivated 5 ovarian cancer cell lines (DOV13, HEYC2, OVCAR2, OVCAR3, and PEO4) to confluence in monolayer cultures. The cell lines were chosen for their ability to form spheroids as well as variation in expression of the stem cell marker CD133 (OVCAR2, OVCAR3 CD133+, others negative). The cells were trypsinized to form single cell suspensions and plated at densities ranging from 1×10^2 to 5×10^3 cells per plate in stem cell-selective media in ultra-low attachment culture dishes. A Zeiss Axio Observer microscope was used for time-lapse photography of the plated cells at 5-minute intervals over a 24-hour period. In addition, single cell suspensions were subjected to flow activated cell sorting to plate one cell in each well of 96-well Costar ultra-low cluster plates to monitor clonal formation of spheroids.

Results: Cells were dissociated and plated under stem cell-selective conditions and 288 images of each cell line were recorded over 24 hours. The cells were found to begin to aggregate within two to four hours, regardless of cell numbers plated. By 24 hours post-plating, there was pronounced aggregation in 5/5 (100%) cell lines with formation of tightly compacted spheroid structures in 4/5 (80%) cell lines. Single cells in the 96-well plates showed no evidence of spheroid formation over a 14-day observation period.

Conclusion: We observed spheroid formation in both CD133 positive and negative ovarian cancer cell lines. Formation occurred due to cellular aggregation rather than clonal expansion from a single progenitor with stem cell properties. Although cellular aggregation may be relevant to ovarian cancer biology, these data suggest that spheroid formation should be viewed with caution if used as a proxy for monoclonal expansion of ovarian cancer stem cells.